Expressional Regulation of Replication Factor C in Adipocyte Differentiation

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Adipocyte differentiation is an ordered multistep process requiring the sequential activation of several groups of adipogenic transcription factors, including CCAAT/enhancer-binding protein-α and peroxisome proliferator-activated receptor-ɣ, and coactivators. In previous reports, we identified that replication factor C 140 (RFC140) protein played a critical role in regulating adipocyte differentiation as a coactivator. Here, we show expressional regulation of RFC140 and small RFC subunit, RFC38, following characterization of gene promoter of RFC140 and RFC38. In addition, RFC140 increases PPARɣ -mediated gene activation, resulting from direct protein-protein interaction of RFC140 and PPARɣ. Taken together, these findings demonstrate that the regulated expression of RFC140 and RFC38 by specific adipocyte transcription factors is required for the adipocyte differentiation process.

Key words : Replication factor C, adipocyte differenation, C/EBP, ATF2, PPAR γ

Introduction

The genetic program of adipogenesis has been studied extensively in vitro [6] with embryonic fibroblasts and pre-adipocyte lines, and in vivo [21,26] with transgenic and knockout mouse models [9], and a paradigm for the cascade of genetic events has emerged. After induction of differentiation, there is a rapid and transient induction of CCAAT/enhancer-binding protein beta (C/EBPβ) and C/EBPδ [4,5]. These transcription factors then activate expression of both C/EBPα and peroxisome proliferator-activated receptor gamma (PPAR γ), which then reinforce each other's expression through a positive feedback loop [20,21]. PPAR γ is a master transcriptional regulator for adipocyte differentiation and is important in regulation of a number of genes involved in fatty acid and glucose metabolism in adipocyte [8,23].

RFC is a five-subunit complex composed of 140-, 40-, 38-, 37-, and 36-kDa subunits [16]. Although RFC140 is primarily responsible for DNA binding, each of the subunits shares a conserved domain that is referred to as the PCNA interaction region [12]. In yeast, each individual subunit is an essential gene product that functions in the RFC complex to "load" PCNA onto DNA prior to genome duplication [15]. Together, DNA polymerase, PCNA, and RFC compose the replicative holoenzyme complex

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[7,19]. But, it can be also suggested likely that RFC140 serves a role in regulating transcription in some manner, either directly by as a cofactor or through a DNA-modifying activity that allows transcription to proceed. The DNA-binding activity of the C/EBPα protein is regulated in the G_1 phase of the hepatocyte cell cycle [11]. From these observations, it is suggested that RFC140 can affect the regulatory role of C/EBPα in the G_1 phase necessary for cell differentiation such as hepatocyte and adipocyte. It is intriguing that C/EBPα, a transcription factor that was reported to induce cellular differentiation of adipocytes, hepatocytes and myelocytes, interacts with a component of the DNA replication apparatus.

Importantly, McGehee and Habener [17] showed that RFC140 was cloned as an important molecule for adipocyte differentiation and increases transcriptionally following the induction of differentiation. Additionally, the treatment of antisense oligonucleotides to RFC140 inhibited adipocyte differentiation [13]. Our earlier report showed that, in adipocyte differentiation, RFC140 expression is increased along with C/EBPα and RFC140 acts as a coactivator of C/EBPα for PPAR γ gene expression [10]. The expression of PPAR γ has been shown to be sufficient to induce growth arrest as well as to initiate adipogenesis in exponentially growing fibroblast cell lines, demonstrating its critical role in the regulation of adipocyte differentiation [1,25]. Here we show that the expressional regulation of RFC140 and small RFC subunit, RFC38, following characterization of gene promoter of RFC140 and RFC38. In addition, RFC140 increases PPAR γ -mediated gene activation, resulting from direct protein-pro-

tein interaction of RFC140 and PPARγ.

Materials and Methods

Database search

Blast searches of GenBank were performed using the BLAST [2] service at the National Center for Biotechnology Information (NCBI, home page www.ncbi.nih.gov). For analyzing transcription factor binding sites in the promoter region, the TRANSFEC service was used.

Cloning of the human RFC140 promoter

The human RFC140 promoter was amplified by PCR twice on human normal spleen PCR Ready genomic DNA. For the primary PCR reaction, primers 5′-CATGGTGCT CCAAGAG-AGCAAGACAGGAGC-3′ (F1) and 5′-ATCCA TTCGCGCCAA CAACTTCTCCCGCGA-3′ (R1) were used. And for the secondary PCR reaction, primers 5′-CGACG CGTGCTGGCTG-CCAGTCTTCTTAAAGC-3′ (F2) and 5′-ATACTCGAGGTGCAAGAAGGCGAGACAGT-3′ (R2) were used. The PCR product was double digested with *MluI* and *XhoI* and cloned into the *MluI*-*XhoI* sites of the pGL3-Basic luciferase reporter plasmid (Promega).

Cloning of the human RFC38 promoter

The human RFC38 promoter was identified using Universal Genome Walker Kit according to the manufacturer's protocol (Clontech). The promoter was amplified by PCR twice. For the primary PCR reaction, RFC38 outer primer 5′-GCCGTCCCAAGGAGCAGGGCCGATAC-TTGT-3′ and AP1 primer 5′-GTAATACGACTCACTATAGGGC-3′ were used. And for the secondary PCR reaction, RFC38 nested primer 5′-CCACCCAGAGGCTCATGG- CAGCTCGAGT TC-3′ and AP2 primer 5′-ACTATAGGGCACGCGTGGT-3′ were used. PCR products were subcloned into pGEM-T Easy vector (Promega) and sequenced using the T7 forward and SP6 reverse primers. Sequencing was performed using the ABIPRISM automated DNA Sequencer model 310 (Applied Biosystem) at the sequencing facility of the Genotech. Promoter DNA sequence was finally cloned at the *SmaI* sites of the pGL3-Basic luciferase reporter vector (Promega).

Cell culture and adipocyte differentiation

3T3-L1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL) and 1% antibiotics.

To induce adipocyte differentiation, cells were grown until 2 days postconfluence (day 0) and then treated for 2 days with growth medium plus MDI (0.5 mM methyl-isobutylxanthine, 1 μM dexamethasone, and 10 μg/ml insulin, all from Sigma). The cells were re-fed with growth medium that contained 5 μg/ml insulin at day 2 and every 2 days thereafter with growth medium alone. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (Gibco BRL), and 1% antibiotics.

Oil red O staining

3T3-L1 cells were washed twice with phosphate-buffered saline (PBS), fixed in 10% formalin, dissolved in PBS for 1 hr, and washed again once with PBS. Cells were then stained with 60% of a filtered oil red O stock solution (0.5 g of oil red O (Sigma) in 100 ml of 99% isopropanol) for 15 min. Cells were washed twice with water for 15 min each time and visualized.

Transient transfection assay

Transient transfections were done in 24-well plates with the standard calcium phosphate method. Cells were incubated with DNA precipitates for 16 hr, washed, and cell extracts were prepared 48 hr after transfection and assayed for relative luciferase and β-galactosidase activities. Basal promoter activity is reported as the activity observed after transfection of the reporter plus an appropriate amount of empty expression vector. In all cases, transfection data represent the mean of three independent experiments.

Mammalian two-hybrid assay

Cells were seeded with growth medium supplemented with 10% fetal bovine serum and 1% antibiotics and co-transfected with expression vectors encoding Gal4-DNA-binding domain fusions (pCMX/Gal4N-, pCMX/Gal4N-RFC140 5′~151, pCMX/Gal4N-RFC140 151~545, pCMX/Gal4N-RFC140 151~3′) and VP16-activation domain fusions (pCMX/VP16-, pCMX/VP16-PPARγ), along with the Gal4-tk-luc reporter plasmid. After 48 h, cells were harvested and the luciferase activity was normalized to the β-galactosidase internal standard. In all cases, transfection data represent the mean of three independent experiments.

Co-immunoprecipitation and Western blot

RFC140 and PPAR were immunoprecipitated from 500 μg

of total cell lysates of differentiation-induced 3T3-L1 cells. The protein content of cell lysates was determined with Bradford reagent (Bio-Rad) using bovine serum albumin (BSA) as standard.

After incubating with 0.5 μg of anti-RFC140 IgG (abcam) and 0.6 μg of anti-PPARγ IgG (Santa Cruz Biotechnology) in a total volume of 100 μl of ice-cold lysis buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 10% Glycerol, 1 mM PMSF, 1 mM DTT, 20 mM NaF, 1 mM EDTA and protease inhibitor) for 2 hr at 4°C, 20 μl of protein G-Sepharose beads and protein A-Sepharose beads (1:1 slurry; Gibco BRL) were added and washed four times with lysis buffer. The precipitates were boiled with an equal volume of 2× Laemmli sample buffer (LSB) at 95°C for 3 min, separated on a 8% SDS-polyacrylamide gel and transfered to PVDF (Immobilon-P) membrane (Millipore). Coprecipitated RFC140 and PPARγ were detected by western blotting with anti-PPARγ IgG and anti-RFC140 IgG in TBS-T (TBS containing 0.04% Tween20) containing 2% skim milk for 1 hr at room temperature. After washing three times with TBS-T, secondary detection was carried out with horseradish peroxidase-conjugated anti-rabbit IgG and anti-goat IgG for 30 min at room temperature. After washing three times with TBS-T, the proteins were visualized by the enhanced chemiluminescent development reagent (Amersham Pharmacia Biotech).

RNA extraction and RT-PCR

The total mRNA of 3T3-L1 cells was extracted using the TRIzol Reagent (Invitrogen). The quantity and purity of the RNA prepared from each sample was determined by the ratio of the optical density at 260 nm to that at 280 nm. Gene expressions were determined by RT-PCR, using a RNA PCR kit incorporating avian myeloblastosis virus-derived RT and Taq DNA polymerase according to the manufacturer's protocol (TaKaRa Biomedicals). The RT reaction mixture was incubated at 42°C for 30 min, heated to 99°C for 10 min and then cooled to 4°C. The PCR mixture contained 2 μl of cDNA, 2.5 mmol/l MgCl$_2$, 1× RNA PCR buffer, 0.2 μ mol/l RFC38 primers or β-actin primers and 2.5 U/100 μl Taq DNA polymerase in a total volume of 80 μl for each reaction. The PCR primers for RFC38 gene amplification were: 5′ -GCCGTCCCAAGGAGCAGGGCCGATACTTGT-3′(F), 5′-CCACCCAGAGGCTCA- TGGCAGCTCGAGTTC-3′(R) and for β-actin gene amplification were : 5′-TCATGAAG-TGTGACGTTGACATCCGT-3′(F′), 5′-CTTAGAAGCATTTG

CGGTGCACGATG-3′(R′). The PCR conditions for RFC38 gene amplification were: 32 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 1 min and extension at 72°C for 1 min in a thermal cycler. For β-actin, 28 cycles of denaturation at 94°C for 30 sec, annealing at 67°C for 1 min, extension at 72°C for 1 min 30 sec. The PCR products were examined by electrophoresis on an agarose gel.

Cell labeling

3T3-L1 cells were cultured in 100 mm dish at density of 1×10^{5}cells/ml in the absence or presence of differentiation inducers, transfected with 5 μg of the indicated expression vectors by standard calcium phosphate method, grown for 24 hr, starved in serum-free Dulbecco's modified Eagle's medium for 30 min, and treated with 100 μCi of [^{32}P]orthophosphate. 3 hr later, cells were washed three times with cold phosphate buffered saline and lysed in lysis buffer (50 mM Hepes (pH 7.5), 150 mM sodium chloride, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1.5 mM magnesium chloride, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 200 mM sodium orthovanadate). Clarified cell lysates (1 mg of proteins) were incubated with anti-RFC140 antibody for 2 hr and protein A-Sepharose beads for 16 hr at 4°C. Immunoprecipitated proteins were washed three times with lysis buffer and the final bead pellets were resuspended in LSB, boiled, centrifuged, and resolved by SDS-PAGE and autoradiography.

Results

Characterization of the human RFC140 promoter

In previous report, mRNA level of RFC 140 was increased during adipocyte differentiation [17]. However, it is unknown that how RFC140 expression is regulated. To understand the mechanism of RFC140 gene expression, the promoter region of RFC140 gene was identified. By searching genome databases, the DNA sequence of the RFC140 was and determined and submitted to the GenBank database with accession number AY600371. The human RFC140 promoter was obtained from human spleen genomic DNA by using PCR. A DNA fragment containing 1.6 kb of 5′- flanking sequence was cut with MluI and XhoI and cloned into the MluI-XhoI sites of the pGL3-Basic luciferase reporter plasmid. This reporter plasmid was used for transient transfection experiments in the human cancer cell line, HeLa cells.

The activation of the 1.6 kb promoter construct was confirmed in the luciferease reporter analysis (Fig. 1A) increased in dose-dependent manner of the transfected promoter amount (data not shown). Sequence analysis of the promoter region revealed that the first 300 bp of the putative RFC140 promoter is very GC-rich, a feature common for promoter regions and several consensus transcription factor binding sites are located in the promoter region but it contains no TATA or CCAAT boxes. A schematic presentation of the RFC140 promoter with binding sites for transcription factors is shown in Fig. 1B.
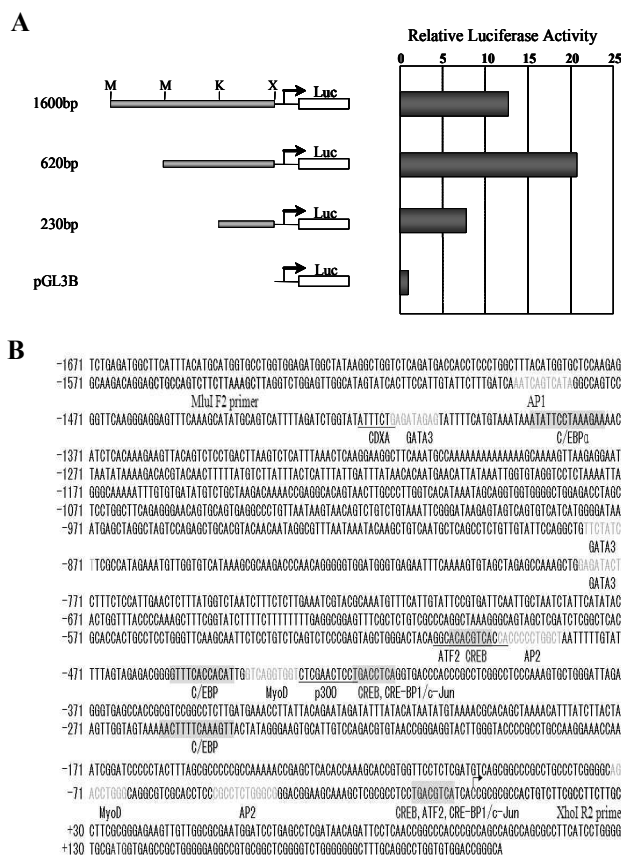
A

B

Fig. 1. Characterization of hRFC140 promoter and the basal activity of hRFC140 promoter. (A) Nucleotide sequence of the human RFC140 promoter from -1662 to +138 is presented. Potential transcription factor binding sites predicted by the TRANSFEC program are represented. Only the sites with a score of >80 are indicated. (B) The human RFC140 promoter containing 1.6 kb of 5′-flanking sequence was generated from human normal spleen genomic DNA and transfected in HeLa cells. The 620bp size construct was generated from 1.6kb RFC140 promoter as template. The smallest fragment generated using the indicated restriction sites (K-*KpnI*, X-*XhoI*) was linked to a pGL3-Basic luciferase reporter. These construct were transfected into the HeLa cells.

Effects of differentiation related transcription factors on hRFC140 promoter activation

Several classes of transcription factors which are related to differentiation can regulate the expression of human RFC140 gene in time dependent manner. CREB and C/EBPβ play an important role in early stage of adipogenesis, and then C/EBPα and PPARγ play a role in middle and late one of adipogenesis [14]. To access which kinds of transcription factors affect the expression of RFC140 gene, transient transfection assay was performed. HeLa cells were transfected with the expression vectors encoding CREB, C/EBPβ, PPARγ, and C/EBPα, along with 1.6 kb RFC140 promoter. The transfection of CREB and C/EBPβ, which are expressed in early stage of adipogenesis, increased the promoter activity of RFC140 by 3- and 2.5-fold, respectively (Fig. 2A, B). In contrast to these, PPARγ, which is expressed in middle stage, did not contribute the increased activation of RFC140 promoter (Fig. 2C). Although C/EBPα has similar transcriptional binding sites of C/EBPβ, it did not increase the promoter activity of RFC140 (Fig. 3D). For the control experiment, C/EBPα increased the promoter activity of PPARγ (Fig. 2E). These results suggest that only CREB and C/EBPβ increase the promoter activity of RFC140 during adipocyte differentiation. Based on the previous study that RFC140 interacts with C/EBPα during adipocyte differentiation [10], the expression of RFC140 might be increased by CREB and C/EBPβ in early stage of adipogenesis, resulting in the enhanced RFC140 interaction with C/EBPα.

Identification of human RFC38 promoter

To further investigate which kinds of transcription factors affect the expression of RFC 38 until mitotic clonal expansion (MCE), the human RFC38 promoter was cloned by using Universal Genome Walker Kit according to the manufacturer's protocol (Clontech). The primary PCR amplification used the outer adaptor primer 5′-GTAATACGACTCACT ATAGGGC-3′ and an outer gene specific primer 5′-GC CGTCCCAAGGAGCAGGGCCGATACTGT-3′. The primary PCR mixture was then diluted and used as a template for a secondary PCR amplification using the nested adaptor primer 5′-ACTATAGGGCACGCGTGGT-3′ and a nested gene specific primer 5′-CCACCCAGAGGCTCATGGCAGCTCG AGTTC-3′. Fig. 3A shows results of primary and secondary Genome Walker PCR. Amplification of each of the Human Genome Walker libraries with the adaptor primers and primers derived from exon 1 of the RFC38 gene gen-
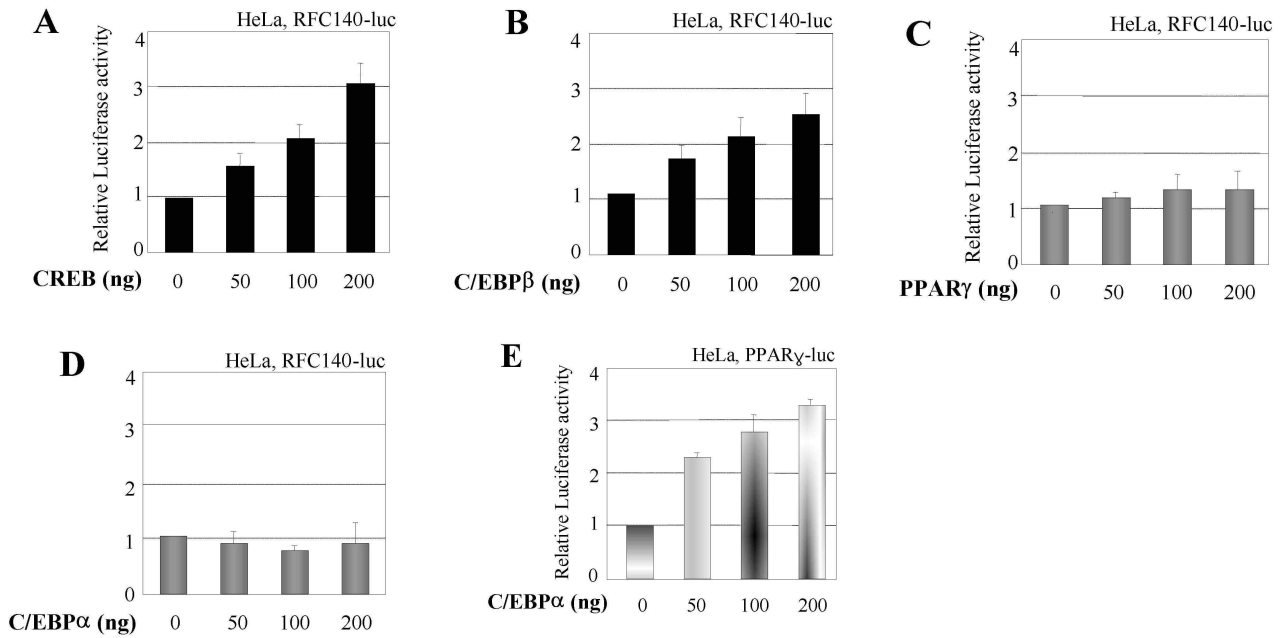
Fig. 2. Effects of differentiation related transcription factors on hRFC140 promoter. (A~D) HeLa cells were transfected with the indicated amounts of expression vectors encoding CREB, C/EBPβ, PPARγ and C/EBPα, along with the RFC140 promoter luciferase reporter. (E) HeLa cells were transfected with the indicated amounts of C/EBPα expression vector, along with the PPARγ 2 promoter luciferase reporter (a gift from Dr. Tae Sung Kim). These data are an average of three independent experiments.
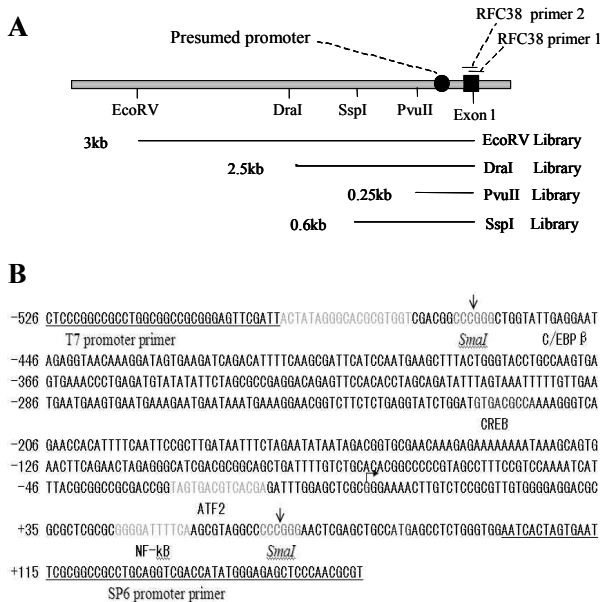

Fig. 3. Cloning and characterization of hRFC38 promoter. (A) Map of the hRFC38 promoter and products of Genome Walker PCR. By using Genome Walker Kit, four PCR products from libraries were obtained. (B) Transcription factor binding sites in the hRFC38 promoter. Nucleotide sequence of the hRFC38 promoter from -526 to +138 is presented. Potential transcription factor binding sites predicted by the TRANSFEC program are represented. Only the sites with a score of >80 are indicated.

erated four major products - 3 kb product with EcoRV library, 2.5 kb product with DraI library, 0.25 kb product with PvuII library and 0.6 kb product with SspI library of the size expected based on the map. Three PCR products except 0.25 kb product were subcloned into pGEM-T Easy vector (Promega) and sequenced using the T7 forward and SP6 reverse primers. Sequencing was performed using the ABIPRISM automated DNA Sequencer model 310 (Applied Biosystem) at the sequencing facility of the Genotech. A DNA fragment containing 0.6 kb 5′- flanking sequence was finally cloned at the SmaI sites of the pGL3 Basic luciferase reporter plasmid (Promega). To test the activity of the promoter, it was transfected in HeLa cells. The activity of the 0.6 kb RFC38 promoter was increased in dose-dependent manner of the transfected promoter amount (data not shown). The nucleotide upstream of the transcription start site was numbered from -1 and several consensus transcription factor binding sites are located in the promoter region (Fig. 3B).

Differential expression of RFC38 with RFC140 during adipocyte differentiation

After induction of differentiation, postconfluent and

growth-arrested 3T3-L1 preadipocytes synchronously reenter the cell cycle and undergo several rounds of MCE at very early stage of differentiation. Recent evidence has established that MCE is required for the progression of the differentiation program [24]. Also, for DNA replication, RFC140 is required to form an active complex with other four small subunits [16]. To access whether RFC140 has a regulatory role of adipogenesis dependent on complex formation or not, the expression pattern of other subunit of RFC was examined by RT-PCR. Because it is known that RFC38 and RFC40 bind RFC140, mRNA levels of these subunits were investigated. Total mRNA was isolated from 3T3-L1 cells on the indicated time after induction of differentiation. In contrast to RFC140, the mRNA levels of RFC38 and RFC40 were maintained to early stage but significantly decreased from 2 days during adipocyte differentiation (Fig. 4A). These results suggest that RFC140 functions as complex until MCE
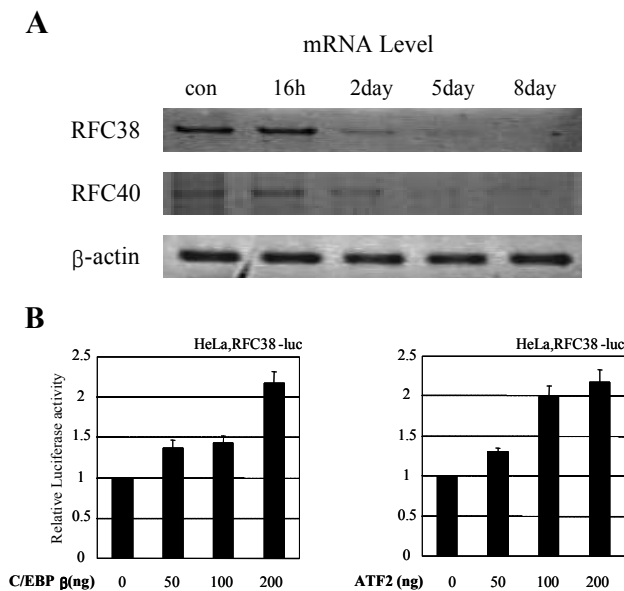

Fig. 4. Expressional regulation of RFC38 and RFC40. (A) The mRNA levels of RFC38 and RFC40. RT-PCR analysis was used to amplify full length open reading frames of RFC38 and RFC40 mRNA. The mRNA levels of RFC38 and RFC40 were significantly decreased during adipogenesis. The β-actin mRNA expression was measured as a loading control. (B) Effects of transcription factors on hRFC38 promoter. The human RFC38 promoter containing 0.6 kb of 5′-flanking sequence was generated from Genome Walker Kit. This construct was transfected into the HeLa cells. HeLa cells were transfected with the indicated amounts of expression vectors encoding C/EBPβ and ATF2, along with RFC38 promoter luciferase reporter.

and as a monomer after middle stage of adipogenesis.

Effects of differentiation related transcription factor on hRFC38 promoter activation

In order to access which kinds of transcription factors affect the expression of RFC38 promoter, transient transfection assay was performed. HeLa cells were transfected with the expression vectors encoding C/EBPβ, and ATF2. As a result, C/EBPβ and ATF2 which are expressed in early stage of adipogenesis increased the promoter activity of RFC38 by 2.2-fold and 2.3-fold, respectively. These results indicate that C/EBPβ can increase the promoter activity of RFC38 as well as RFC140 earlier in the differentiation program, i.e., during MCE.

The interaction of RFC140 and PPAR in adipocyte differentiation

The protein level of RFC140 was increased after induction of adipocyte lineage differentiation of 3T3-L1 cells by inducers [17]. The transcription factor PPARγ is known to be both necessary and sufficient for adipocyte differentiation. These results suggest the possibility that PPARγ has functional interaction with RFC140 in the adipocyte differentiation procedure. To examine whether the protein interaction between RFC140 and PPARγ would be found in the differentiation process of adipocytes, transient transfection assay was performed. HeLa cells were transfected with the indicated amounts of expression vectors encoding RFC140 and PPARγ, along with the PPRE promoter linked to the luciferase gene. 48 hr after transfection, cells were harvested for luciferase assay. Whereas the transfection of RFC140 alone did not significantly affect basal reporter activity, the transfection of PPARγ alone stimulated the promoter activity of PPRE by 18-fold. When cells were cotransfected with PPARγ and RFC140, the promoter activity of PPRE was increased by 25-and 40-fold in RFC140 dose dependent manner (Fig. 5A). To confirm the interaction of two factors, co-immunoprecipitation was performed. As shown in Fig. 5B, RFC140 was detected in the co-immunoprecipitated protein fractions by anti-PPARγ antibody using differentiation-induced cell lysates at the 3rd and 5th days after inducer treatment. Reversely, PPARγ was detected in the co-immunoprecipitated protein fractions by anti-RFC140 antibody using differentiation-induced cell lysates at the 3rd and 5th days after inducer treatment. This interaction was not found in undifferentiated cell lysates and in
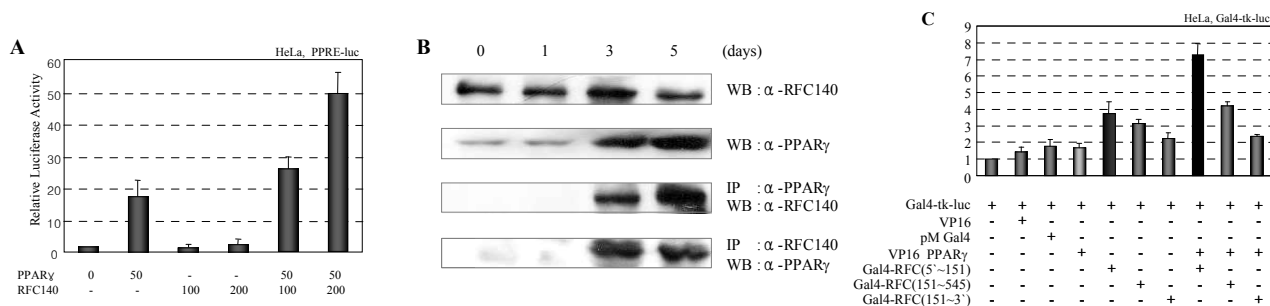
Fig. 5. The function of RFC140 in PPARγ -dependent transactivation activity. (A) HeLa cells were transfected with the indicated amounts of expression vectors encoding RFC140 and PPARγ, along with the PPRE-luciferase plasmid. 48 hr after transfection, cells were harvested for luciferase activities. This data is an average of three independent experiments. (B) The interaction of RFC140 and PPARγ in adipocyte differentiation. 3T3-L1 cells were treated with differentiation inducers (MDI) and harvested after 1, 3 and 5 days. RFC140 was immunoprecipitated (IP) from the total cell lysates of differentiation-induced 3T3-L1 cells by incubation with anti-RFC140 IgG. Antibody complexes were captured on protein G-Sepharose. The beads were washed four times with lysis buffer. Finally, Western blotting was carried out with anti-PPARγ antibody (Third pannel). PPARγ was immunoprecipitated (IP) from the total cell lysates of differentiation-induced 3T3-L1 cells by incubation with anti-PPARγ IgG. Antibody complexes were captured on protein A-Sepharose. The beads were washed four times with lysis buffer. Finally, Western blotting was carried out with anti-RFC140 antibody (Fourth pannel). (C) The binding domain of RFC140 with PPARγ. The mammalian expression plasmids encoding Gal4-RFC140 (5'~151), Gal4-RFC140 (151~545), Gal4-RFC140 (151~3') and VP16-PPARγ were transfected into HeLa cells, as indicated. Then, 48 hr after transfection, cells were harvested for luciferase activities. This data is an average of three independent experiments.

the first day lysates after differentiation induction. Lastly, to address the binding domain of RFC140 with PPARγ, mammalian two-hybrid assay was performed. As shown in Fig. 5C, coexpression of Gal4-RFC140 NH2-terminal region and VP16-PPARγ enhanced Gal4-tk-luc-dependent transactivation, but coexpression of Gal4-RFC140 COOH-terminal region and VP16-PPARγ didn't affect the activity of Gal4-tk-luc. These results indicate that RFC140 can interact with PPARγ through the NH2-terminal region and function as co-activator.

Discussion

Adipocyte differentiation is a complex process accompanied by coordinated changes in morphology, hormone sensitivity, and gene expression. These changes are regulated by several transcription factors such as C/EBPs, PPAR γ, and ADD1/SREBP1c [22]. These transcription factors are induced at different stages of adipocyte differentiation and functionally interact with each other to conduct adipogenesis and lipogenesis by regulating gene expression [3]. RFC is a five-subunit complex composed of 140-, 40-, 38-, 37- and 36-kDa subunits [18]. Although RFC140 is primarily responsible for DNA replication and repair, it has been demonstrated that RFC140 is important to regulate gene expression.

In the previous paper, it was demonstrated that mRNA level of RFC 140 has increased during adipocyte differentiation. However, the molecular mechanism of RFC140 gene expression during adipocyte differentiation is largely unknown.

In this study, to investigate the mechanism of RFC140 gene expression, the promoter region of RFC140 gene was identified. Through the transient transfection assay, it was demonstrated that CREB and C/EBPβ bind to the promoter of RFC140 gene in early stage of adipocyte differentiation and activate the expression of RFC140 gene in dose-dependent manner (Fig. 2A and B), but PPARγ and C/EBPα expressed in middle and late stage of adipocyte differentiation do not increase the promoter activity (Fig. 2C and D). To access whether RFC140 functions as complex or monomer during adipogenesis, mRNA levels of RFC38 and RFC40 were investigated and elucidated that they are maintained until MCE but significantly decreased during adipocyte terminal differentiation (Fig. 4A). Also, it was demonstrated that C/EBPβ and ATF2 bind to the promoter region of RFC38 gene in early stage of adipocyte differentiation and activate the expression of RFC38 in dose-dependent manner during mitotic clonal expansion stage. However, to know the regulatory mechanism of RFC140 by CREB and C/EBPβ in detail, Chromatin Immunoprecipitation (ChIP) should be

performed with antibodies directed against transcription factors after identifying of DNA regions which are bound by CREB and C/EBPβ.

In previous report, when the differentiation inducer, insulin was treated in 3T3-L1 cells, RFC140 was phosphorylated by activated CaMKII and the phosphorylated RFC140 largely increased the coactivator activity of RFC140 on C/EBPα-mediated transactivation. Thus, it will be interesting to examine how the coactivator activity of RFC140 can be increased by phosphorylation. As several possibilities, RFC140 can increase the DNA binding activity of C/EBPα

or recruit other coactivators to the region well. Another possibility is that it may serve a role in regulating transcription through a DNA modifying activity that allows transcription to proceed, because it has a helicase like domain.

The function of PPARγ is especially critical, since many of the genes involved in adipogenesis, as well as glucose homeostasis, are activated by this nuclear hormone receptor. On the previous data, it was shown that RFC140 interacts with C/EBPα during adipogenesis. So, it was assumed that RFC140 might be able to interact with other factors related adipocyte differenciation. By co-immunoprecipitation analysis, it was demonstrated that the general DNA replication factor RFC140 physically interact with an important transcription factor PPARγ. Through the interaction RFC140 with PPARγ as well as C/EBPα, it was considered that RFC140 is a major factor in adipocyte differentiation not by interacting a specific "only one" factor but by associating many factors related adipocyte differentiation. In short, it seems that RFC140 functions broadly. For the further study, it is necessary to investigate the interaction with other factors-SREBP and LXR, because they are associated in adipocyte differentiation. Lastly, to address the binding domain of RFC140 with PPARγ, mammalian two-hybrid assay was performed and showed the possibility that RFC140 interacts with PPARγ through the NH2-terminal domain.

In summary, the interpretation of results in the context of the cascade model of adipocyte differentiation can be supposed to explain the functional regulation of RFC140. First, the expression of RFC140 is regulated by CREB and C/EBPβ and that of RFC38 is increased by C/EBPβ and ATF2 in early stage of adipogenesis. RFC140 functions as complex until MCE and as a monomer after middle stage of adipogenesis. Second, RFC140 is phosphorylated by activated CaMKII and the phosphorylated RFC140 largely in-

creases the coactivator activity of RFC140 on C/EBPα-mediated transactivation. Third, RFC140 plays an important role by interacting with PPARγ, which is a key regulator of adipocyte differentiation. RFC140 may enhance the function of target gene enhancesome by juxtaposing components of the transcriptional machinery in a more favorable orientation, and may also play a role in recruiting transcriptional coactivators.

Acknowledgement

References

1. Altiok, S., M. Xu, and B. M. Spiegelman. 1997. PPARγ induces cell cycle withdrawal: inhibition of E2F/DP DNA-binding activity via down-regulation of PP2A. *Genes Dev.* **11,** 1987-1998.
2. Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25,** 3389-402.
3. Brun, R. P., J. B. Kim, E. Hu, and B. M. Spiegelman. 1997. Peroxisome proliferator-activated receptor gamma and the control of adipogenesis. *Curr. Opin. Lipidol.* **8,** 212-218.
4. Cao, Z., R. M. Umek, and S. L. McKnight. 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev.* **5,** 1538-1552.
5. Clarkson, R. W., C. M. Chen, S. Harrison, C. Wells, G. E. Muscat, and M. J. Waters. 1995. Early responses of trans-activating factors to growth hormone in preadipocytes: differential regulation of CCAAT enhancer-binding protein-beta (C/EBP beta) and C/EBPdelta. *Mol. Endocrinol.* **9,** 108-120.
6. Dani, C., A. G. Smith, S. Dessolin, P. Leroy, L. Staccini, P. Villageois, C. Darimont, and G. Ailhaud. 1997. Differentiation of embryonic stem cells into adipocytes in vitro. *J. Cell Sci.* **110,** 1279-1285.
7. DePamphilis, M. L. 1993. Eukaryotic DNA replication: anatomy of an origin. *Annu. Rev. Biochem.* **62,** 29-63.
8. Ferre, P. 2004. The biology of peroxisome proliferator-activated receptors: relationship with lipid metabolism and insulin sensitivity. *Diabetes* **53,** 43-50.
9. Guerra, C., P. Navarro, A. M. Valverde, M. Arribas, J. Brüning, L. P. Kozak, C. R. Kahn, and M. Benito. 2001. Brown adipose tissue - specific insulin receptor knockout shows diabetic phenotype without insulin resistance. *J. Clin. Invest.* **108,** 1205-1213.
10. Hong, S., S. J. Park, H. J. Kong, J. D. Shuman, and J. Cheong. 2001. Functional interaction of bZIP proteins and the large subunit of replication factor C in liver and adipose cells.

J. Biol. Chem. **276**, 28098-28105.

11. Iakova, P., S. S. Awad, and N. A. Timchenko. 2003. Aging reduces proliferative capacities of liver by switching pathways of C/EBPα growth arrest. *Cell* **113**, 495-506.

12. Kolpashchikov, D. M., P. Hughes, A. Favre, G. Baldacci, and O. I. Lavrik. 2001. Localization of the large subunit of replication factor C near the 5′ end of DNA primers. *J. Mol. Recognit.* **14**, 239-244.

13. Lyle, R. E., J. F. Habener, and R. E. Mcgehee. 1996. Antisense oligonucleotides to differentiation-specific element binding protein (DSEB) mRNA inhibit adipocyte differentiation. *Biochem Biophys. Res. Commun.* **228**, 709-715.

14. MacDougald, O. A. and M. D. Lane. 1995. Adipocyte differentiation. When precursors are also regulators. *Curr. Biol.* **5**, 618-621.

15. Majka, J. and P. M. Burgers. 2003. Yeast Rad17/Mec3/Ddc1: A sliding clamp for the DNA damage checkpoint. *Proc. Natl. Acad. Sci. USA* **100**, 2249-2254.

16. Maruyama, T., A. Farina, A. Dey, J. Cheong, V. P. Bermudez, T. Tamura, S. Sciortino, J. Shuman, J. Hurwitz, and K. Ozato. 2002. A mammalian bromodomain protein, Brd4, interacts with replication factor C and inhibits progression to S phase. *Mol. Cell Biol.* **22**, 6509-6520.

17. McGehee, R. E. Jr. and J. F. Habener. 1995. Differentiation-specific element binding protein (DSEB) binds to a defined element in the promoter of the angiotensinogen gene required for the irreversible induction of gene expression during differentiation of 3T3-L1 adipoblasts to adipocytes. *Mol. Endocrinol.* **9**, 487-501.

18. Mossi, R. and U. Hübscher. 1998. Clamping down on clamps and clamp loaders the eukaryotic replication factor C. *Eur. J. Biochem.* **254**, 209-216.

19. Podust, V. N., N. Tiwari, S. Stephan, and E. Fanning. 1998. Replication factor C disengages from proliferating cell nuclear antigen (PCNA) upon sliding clamp formation, and PCNA itself tethers DNA polymeraseδ to DNA. *J. Biol. Chem.* **273**, 31992-31999.

20. Prusty, D., B. H. Park, K. E. Davis, and S. R. Farmer. 2002. Activation of MEK/ERK signaling promotes adipogenesis by enhancing peroxisome proliferator-activated receptor gamma (PPARγ) and C/EBPα gene expression during the differentiation of 3T3-L1 preadipocytes. *J. Biol. Chem.* **277**, 46226-46232.

21. Rosen, E. D., P. Sarraf, A. E. Troy, G. Bradwin, K. Moore, D. S. Milstone, B. M. Spiegelman, and R. M. Mortensen. 1999. PPARγ is required for the differentiation of adipose tissue *in vivo* and *in vitro*. *Mol. Cell* **4**, 611-617.

22. Spiegelman, B. M. and J. S. Flier. 1996. Adipogenesis and obesity: rounding out the big picture. *Cell* **87**, 377-389.

23. Tamori, Y., J. Masugi, N. Nishino, and M. Kasuga. 2002. Role of Peroxisome Proliferator-Activated Receptor- γ in maintenance of the characteristics of mature 3T3-L1 adipocytes. *Diabetes* **51**, 2045-2055.

24. Tang, Q. Q., T. C. Otto, and M. D. Lane. 2003. Mitotic clonal expansion: a synchronous process required for adipogenesis. *Proc. Natl. Acad. Sci. USA* **100**, 44-49.

25. Tontonoz, P., E. Hu, and B. M. Spiegelman. 1995. Regulation of adipocyte gene expression and differentiation by peroxisome proliferator activated receptor γ. *Curr. Opin. Genet. Dev.* **5**, 571-576.

26. Yajima, Y., M. Sato, M. Sumida, and S. Kawashima. 2003. Mechanism of adult primitive mesenchymal ST-13 preadipocyte differentiation. *Endocrinology* **144**, 2559-2565.

초록 : 지방세포분화에서의 replication factor C 단백질의 발현조절

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지방세포 분화 과정 중에 key regulator로서 기능을 하는 여러 전사조절인자(PPARγ, C/EBPα, SREBP, LXR)가 동정되었고, 주로 DNA 복제나 DNA 수선 단계에서 중요한 역할을 한다고 밝혀진 복제 조절인자인 RFC140이 지방세포 분화에도 중요한 인자임이 밝혀졌다. 이 연구에서 우리는 RFC140과 RFC38에 대한 발현조절을 확인하였으며, RFC140이 PPARγ와의 단백질-단백질 결합을 통하여 PPARγ에 의해 조절되는 유전자의 발현을 증가시킴을 확인하였다. 이러한 결과들은 특이적인 지방세포 전사인자에 의해 발현이 조절되는 RFC140과 RFC38이 지방세포의 분화과정에 필수적임을 제시한다.